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Agents for the Immunotherapy of Tumoral Diseases

The present invention relates to agents suitable for the immunotherapy of tumoral diseases. These agents are tumor cells, a tumor cell library containing them and vaccines containing said tumor cells. The invention also relates to a method for producing the tumor cells and to the use of these cells and the vaccines and the tumor cell library.

The most varying methods are used or treating tumoral diseases. Primary tumors are often removed by means of surgery, and the patients are subjected to a follow-up in the form of a chemotherapy and/or a radiation therapy. The follow-up shall serve for destroying residual tumor cells. Immunotherapy methods are also employed in which tumor cells obtained from primary tumors are manipulated and returned to the patients. This shall serve for sensitizing the immune system to the tumor cells so as to prevent subsequent formation of metastases. However, the immunotherapy methods do not yet show the desired results. The sensitization of the immune system often fails to suffice, so that tumor cells remain unidentified and metastases may develop. Immunotherapy methods also require the use of patient-inherent tumor cells for the treatment. This is very costly and time-consuming. Immunotherapy often cannot be carried out at all because it is not possible to collect sufficient tumor cells from the individual patient.

Therefore, it is the object of the present invention to provide an agent by means of which immunotherapy of tumoral diseases can be carried out, the above drawbacks being avoided.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on Applicant's insights regarding the correlation between the expression of MHC ("major histocompatibility complex") I, II genes in tumor cells and the immunogenicity thereof. MHC I genes are also referred to as HLA-A, HLA-B and HLA-C genes. Furthermore, MHC II genes are also referred to as HLA-Dr, HLA-DQ, and HLA-DP genes. Applicant recognized that the expression of MHC I and/or MHC II genes is disturbed in tumor cells. In particular, he found that many tumor cells do not express MHC II genes. He also found that because of the disturbed expression of the MHC I and/or MHC II genes and the accompanying lack of corresponding gene products on the surface of tumor cells, the tumor antigens present on this surface are not recognized as foreign antigens by the immune system and therefore the tumor cells are not destroyed. On the other hand, Applicant discovered that tumor cells which have a combination of MHC I and MHC II genes, occurring in humans, and also express it, show a high degree of immunogenicity. Such combinations are particularly those indicated in Table I. He also discovered that the immunogenicity of such tumor cells can even be increased if they also express co-stimulatory molecules and/or cytokines.

According to the invention Applicant's insights are utilized to provide tumor cells with a combination of MHC I and MHC II genes, occurring in humans, which genes are expressed.

The expression "tumor cells" comprises tumor cells of any tumor occurring in man. Examples of tumors comprise mammary carcinomas, anogenital carcinomas, lung carcinomas, colon carcinomas, brain tumors, gastric carcinomas, bladder carcinomas, liver cell carcinomas and melanoma.

The tumor cells may be freshly isolated or be present in culture. They may also be present as such or in a cell aggregate, e.g. (primary) tumor or metastasis.

The expression "combination of MHC I and MHC II genes" comprises any combination of MHC I and MHC II genes which may occur in humans. In particular, the combination is selected from the combinations indicated in Table I.

The term "expressed genes" refers to the fact that the combination of MHC I and MHC II genes is expressed. This can be achieved by common methods. It is favorable to initially subject the tumor cells and optionally other cells, e.g. lymphocytes, from the same patient to tissue typing so as to determine which of the MHC I and/or MHC II genes show a disturbed expression. Tissues may be typed e.g. by serological methods, as known from the "11th International Histocompatibility Workshop". The disturbed expression of the MHC I and/or MHC II genes may then be compensated by transfection of corresponding exogenous genes, optionally present on expression vectors, in the tumor cells. Examples of expression vectors comprise pUHD10-I, pRcRSV, pBSK, RSV.5 hygro, pBJ and B45-neo, and examples of transfection methods are calcium phosphate co-precipitation, electroporation, lipofection, DOTAP liposomes and retroviral transfection. The expression of transfected MHC I and/or MHC II genes may be stable or transient, stable ones being preferred. The expression may be detected by common methods, e.g. serological methods, see above.

In a preferred embodiment, the above tumor cells also have one or several genes which code for co-stimulatory molecules and/or cytokines, which are expressed. Examples of co-stimulatory molecules are B7, such as B7-1 or B7-2, and CD44. Examples of cytokines comprise interleukins, such as IL-2, GM-CSF, TNF-α and interferon-γ. The presence of the addressed genes in the tumor cells and the expression of the genes may be achieved as usual. Reference is made to the above statements.

A further subject matter of the present invention relates to a method of producing the above tumor cells. Such a method comprises the steps of:

(a) tissue typing of tumor cells,
(b) transfection of the tumor cells using MHC I and/or MHC II genes so as to obtain a combination of these genes, occurring in humans, and
(c) selection on the tumor cells which express the MHC I and MHC II genes.

The expression "tissue typing of tumor cells" comprises any method serving for determining the expression of MHC I and MHC II genes. Reference is made to the above statements. It may be favorable to subject cells, e.g. lymphocytes, of the same patient from which the tumor cells originate to tissue typing. This facilitates a detection of the combination of MHC I and MHC II genes suitable for this patient.

The expression "transfection of tumor cells" comprises any method by which MHC I and/or MHC II genes can be transfected in tumor cells. Reference is made to the above statements.

The expression "selection on tumor cells" comprises any methods serving a selection on tumor cells which express MHC I and MHC II genes. Reference is made to the above remarks.

In a preferred embodiment, the tumor cells are also transfected with one or several genes coding for co-stimulatory molecules and/or cytokines and selected for the expression of these genes. Reference is made to the above remarks.

A further subject matter of the present invention relates to a tumor cell library comprising the above tumor cells. The tumor cells preferably originate from any human tumor and comprise any combination of MHC I and MHC II genes occurring in man. It is particularly preferred that the tumor cells

comprise the combinations of MHC I and MHC II genes, indicated in Table I.

A further subject matter of the present invention relates to a vaccine comprising the above tumor cells and conventional auxiliary agents, e.g. buffers, carriers and diluents. The vaccine preferably contains tumor cells of different tumors with equal combination of MHC I and MHC II genes each.

The present invention provides tumor cells which express a combination of MHC I and MHC II genes, the combination occurring in a human being. In particular, such a combination occurs in many humans. Thus, the tumor cells according to the invention represent a product which cannot be administered merely to a certain human being but can be given to many humans. In addition, the tumors originate from any human tumor. In so far, the present invention is not limited to the treatment of a certain tumor but can be used for a wide range of tumors.

By means of the present invention it is possible to offer immunotherapy to patients suffering from a tumoral disease. The major advantage of this therapy is that it can be carried out rapidly. Having determined the kind of tumor and its typing or other cells originating from the same patient, suitable tumor cells according to the invention can be selected, e.g. from the tumor cell library, and be administered to the patient. Before administering the tumor cells, it is favorable to prevent them from replicating by certain measures, such as irradiation.

Furthermore, the present invention enables prophylactic steps to be taken against all kinds of tumors. For this purpose, it is merely necessary to type the tissue of cells from the human to be treated and then give the latter a suitable vaccine according to the invention.

Thus, the present invention is a break-through for the immunotherapy of tumoral diseases.

The invention is explained by the below example.

Example: Preparation of tumor cells according to the invention

(A) Establishing tumor cells of a melanoma patient

The tumor of a melanoma patient is removed, comminuted and placed in several cell culture bottles containing DMEM medium. After 48 hours of culturing (37°C, 5 % CO_2), the medium is exchanged. Five cell culture bottles are selected after 1-2 weeks, which are treated independently.

(B) Tissue typing of lymphocytes and tumor cells of a melanoma patient

1. Making lymphocytes and tumor cells ready for tissue typing.

Blood is withdrawn from melanoma patients from (A). This blood is supplied with an anti-coagulant and diluted with the same volume of HBSS solution. The blood is filled into small tubes which already contain ficoll. The tubes are centrifuged at 800 g for 20 min. The resulting interphase is removed, resuspended in HBSS solution, and centrifuged at 500 g for 5 min. The resulting lymphocytes are counted and standardized for tissue typing as a solution having a concentration of 1-2 x 10^6/ml.

The established tumor cells from (A) are standardized in equal concentration.

2. Serologic determination of HLA molecules on lymphocytes and tumor cells

Polystyrene plates are used which are coated with antisera against HLA-A, HLA-C, HLA-B, HLA-DR, HLA-DQ and/or HLA-DP. 1 µl of the lymphocyte solution or tumor cell solution from

(B) 1. is added to each of these plates. The plates are incubated at 22°C for 30 min. before 5 µl fresh complement are added each. Then, the plates are incubated at 22°C for 60 min. before 1 µl of an acridine orange/ethidium bromide cocktail and 1 µl of quencher solution are added each. The plates are allowed to stand at room temperature for 4 hours. Positive samples are shown by the development of fluorescent staining.

It shows that the lymphocytes comprise the following HLA molecules:
A*01; Cw*07; B*08; DRB1*03; DQA1*02; DQB1*02; DPA1*02; DPB1*02.

The tumor cells, however, only have the following HLA molecules:
A*01; Cw*07; B*08.

3. Determining the HLA genes of lymphocytes

RNA is isolated from the lymphocytes from (B) 1. and subjected to reverse transcription. The resulting cDNA is subjected to a PCR method which uses primer groups that are selected in accordance with the HLA molecules from (B) 2. In particular, the following primers are used:

A*01:	forward:	CGA CGC CGC GAG CCA GAA
	reverse:	AGC CCG TCC ACG CAC CG
Cw*07:	forward:	GGA CCG GGA GAC ACA GAA C
	reverse:	CGC ACG GGC CGC CTC CA
B*08:	forward:	GAC CGG AAC ACA CAG ATC TT
	reverse:	CCG CGC GCT CCA GCG TG
DRB1*03:	forward:	GAC GGA GCG GGT GCG GTA
	reverse:	CTG CAC TGT GAA GCT CTC CA
DQA1*02:	forward:	CGA GTT TTA CGG TCC CTC TGG C

reverse: CTC ATT GGT AGC AGC GGT AGA GTT GG

DQB1*02: forward: GTG CGT CTT GTG AGC AGA AG
reverse: CGT GCG GAG CTC CAA CTG

DPA1*02: forward: CCC GCT CTG GTT TGA TTT AT
reverse: CAC TTC GCA TCT ATG CGA

DPB1*02: forward: AGG ACA GAA CTC GGT ACT AGG A
reverse: TGA ATC CCC AAC CCA AAG TCC CC

The PCR conditions are as follows: 24 cycles: 1 min, 94°C; 45 sec, 65°C; 2 min, 72°C. Final cycle: 1 min, 94°C; 45 sec, 65°C; 10 min, 72°C.

The amplified DNA is cleaved by the restriction enzymes SalI and HindIII and inserted in the correspondingly cleaved vector M13 mp18 or M13 mp19. Recombinant DNA molecules are used for the transformation of *E. coli* JM109. Resulting clones are subjected to a screening method by means of hybridization with the amplified DNA. Positive clones are subjected to sequencing.

It shows that the HLA molecules from (B) 2. are encoded by the following HLA genes:

A*0101; Cw*0701; B*0801; DRB1*0301; DQA1*0201; DQB1*0201; DPA1*0201; DPB1*0201.

4. Isolation of HLA-D genes from lymphocytes and transfection thereof in tumor cells

Blood is withdrawn from the melanoma patient from (A). This blood is supplied with an anti-coagulant and diluted with eight times an excess of RCL buffer. The blood is centrifuged in a microcentrifuge for 30 sec. The pellet is taken up with RCL buffer and centrifuged. Having repeated this step several times, the pellet is dissolved in NLB buffer and incubated with proteinase K at 63-65°C for 1 hour

and at 95°C for 10 min. The solution is centrifuged in a microcentrifuge for 60 sec and the pellet is discarded. The supernatant contains the DNA from the lymphocytes.

This DNA is subjected to a PCR method which employs the primers used in (B) 2. for the HLA-D genes, DRB1*03, DQA1*02, DQB1*02, DPA1*02 and/or DPB1*02.

The PCR conditions are as follows: 30 cycles; 30 sec, 98°C; 60 sec, 55°C; 105 sec, 72°C. Final cycle: 7 min., 72°C.

Samples of the amplified DNA are separated on a 1 % agarose gel by means of electrophoresis. This yields fragments of 216 bp for DRB1*03, of 219 bp for DQA1*02/DQB1*02, and of 245 bp for DPA*02/DPB1*02.

The amplified DNA is cleaved by the restriction enzymes SalI and HindIII and inserted in the correspondingly cleaved expression vector B45-neo. Recombinant DNA molecules are used for the transformation of *E. coli* JM109 or DH5F'α. Resulting clones are subjected to a screening method by means of hybridization using the amplified DNA. Positive clones are confirmed by sequencing.

These clones are used for the transfection of the tumor cells from (A). The tumor cells are trypsinated, and electroporation is carried out with 400 V and 490 µ FD. The tumor cells are selected with G418 (400 - 100 µg/ml) for 4 weeks before they are subjected to "fluorescence activating cell sorting" (FACS). Tumor cells are obtained which express the following HLA molecules:

A*0101; Cw*0701; B*0801; DRB1*0301; DQA1*0201; DQB1*0201; DPA1*0201; DPB1*0201.

5. Transfection of tumor cells with genes coding for co-stimulatory molecules and/or cytokines

cDNAs which code for CD44, IFN-γ and/or GM-CSF are obtained from Invitrogen company. The cDNAs are inserted in the expression vectors RSV.5 hygro (blunt/BamHI), pUHD10-1 (XmnI) and/or pBSK (BamHI). Recombinant DNA molecules are used for transforming *E. coli* JM109 or DH5'α. Resulting clones are subjected to a screening method by means of hybridization with the cDNAs. Positive clones are confirmed by sequencing.

These clones are used for transfecting the tumor cells obtained in (B) 4. The transfection is carried out with DOTAP liposomes according to the instructions from the manufacturer Boehringer Mannheim. The transfected tumor cells are screened by FACS and/or RT-PCR. Tumor cells are obtained which express the following HLA molecules and co-stimulatory molecules as well as cytokines:

A*0101; Cw*0701; B*0801; DRB1*0301; DQA1*0201; DQB1*0201; DPA1*0201; DPB1*0201; IFN-γ; DC44; GM-CSF.

Table I

Frequent HLA Combinations

People	HLA-A	HLA-C	HLA-B	HLA-DR	HLA-DQ	HLA-DP	Frequency
Cornish	A*-0101	Cw*-0701	B*-0801	DR*-0301	DQ*-0201	DP*-0101	8.4%
German	A*-0101	Cw*-0701	B*-0801	DR*-0301	DQ*-0201	DP*-0401	4.8%
German	A*-0101	Cw*-0701	B*-0701	DR*-1501	DQ*-0101	DP*-0401	2.5%
USA	A*-1001	Cw*-0701	B*-0801	CR*-0301	DQ*-0201	DP*-0401	4.3%
Canadian	A*-0101	Cw*-0701	B*-0801	DR*-0301	DQ*-0201	DP*-0101	5.1%
Australian	A*-0101	Cw*-0701	B*-0801	DR*-0301	DQ*-0201	DP*-0101	7.6%
Japanese	A*-2401	CBL	B*-5201	DR*-1501	DQ*-0101	DP*-0901	8.2%
Japanese	A*-3301	CBL	B*-4401	DR*-1302	DQ*-0101	DP*-0401	4.9%
Indian	A*-2401	CBL	B*-6101	DR*-1501	DQ*-0101	DP*-0402	4.1%
Thais	A*-0201	Cw*-1101	B*-4601	DR*-0901	DQ*-0301	DP*-0401	4.5%
Taiwan	A*-2401	Cw*-0701	B*-3901	DR*-1201	DQ*-0701	DP*-1301	10.4%
Inuit	A*-2401	CBL	B*-4801	DR*-0401	DQ*-0701	DP*-0201	9.4%
Singapore	A*-0201	Cw*-1101	B*-4601	DR*-0901	DQ*-0301	DP*-0401	7.2%
Maori	A*-0201	Cw*-0101	B*-5501	DR*-1201	DQ*-0701	DP*-0101	8.1%
Bushman	A*-3001	CW*-0401	B*-5801	DR*-1301	DQ*-0101	DP*-0401	8.2%
North Am.-Negroid	A*-3601	CW*-0401	B*-5301	DR*-1101	DQ*-0101	DP*-0101	1.1%

Bas-que	A*-2901	CBL	B*-4401	DR*-0701	DQ*-0201	DP*-0201	5.4%
Java-nese	ABL	CBL	B*-6201	DR*-1201	DQ*-0701	DP*-0401	8.2%
Mon-goli-an	A*-3001	Cw*-0601	B*-1301	DR*-0701	DQ*-0201	DP*-0201	4.0%
Ura-lic	A*-1101	CW*-0401	B*-3501	DR*-0301	DQ*-0201	DP*-0101	3.1%